

CROSS-REFERENCE TO RELATED APPLICATION

5 This is a continuing application under 35USC120 of US Patent Application Serial No. 08/553,727, filed Oct 23, 1995.

INTRODUCTION

Field of the Invention

10 The field of this invention is a novel human kinase involved in tumor necrosis factor signal transduction and its use in drug screening.

Background

15 Tumor necrosis factor (TNF) is an important cytokine involved in the signaling of a number of cellular responses including cytotoxicity, anti-viral activity, immun.-regulatory activities and the transcriptional regulation of a number of genes. The TNF receptors (TNF-R1 and TNF-R2) are members of the larger TNF receptor superfamily which also includes the Fas antigen, CD27, CD30, CD40, and the low affinity nerve growth factor receptor. Members of this family have been shown to participate in a variety of biological properties, including programmed cell death, antiviral activity and activation of the transcription factor  
20 NF- $\kappa$ B in a wide variety of cell types.

Accordingly, it is desired to identify agents which specifically modulate transduction of TNF receptor family signaling. Unfortunately, the components of the signaling pathway remain largely unknown; hence, the reagents necessary for the development of high-  
25 throughput screening assays for such therapeutics are unavailable. Elucidation of TNF receptor family signal transduction pathways leading to NF- $\kappa$ B activation would provide valuable insight into mechanisms to alleviate inflammation. In particular, components of this pathway would provide valuable targets for automated, cost-effective, high throughput drug screening and hence would have immediate application in a broad range of domestic and  
30 international pharmaceutical and biotechnology drug development programs.

Relevant Literature

Stanger et al. (1995) Cell 81, 513-523 report the existence of a Receptor Interacting Protein (RIP) and its functional expression. VanArsdale and Ware (1994) J Immunology  
35 153:3043-3050 describe proteins associated with TNF-R1. The cloning and amino acid

sequencing of TNF-R1 is disclosed in Schall et al (1990) Cell 61, 361 and Loetscher et al (1990) Cell 61, 351; the identification of a "death domain" in TNF-R1 is disclosed in Tartaglia et al. (1993) Cell 74:845-853. The cloning and amino acid sequence of a TNF-R associated death domain protein (TRADD) is described by Hsu et al. (1995) Cell 81, 495-504. The cloning and amino acid sequence of the Fas antigen is disclosed in Itoh et al (1991) Cell 66, 233-243. For a recent review, see Smith et al. (1994) Cell 76:959-962 and Vandenabelle et al. (1995) Trends Cell Biol. 5, 392-399.

## SUMMARY OF THE INVENTION

The invention provides methods and compositions relating to a human Receptor Interacting Protein (hRIP). The compositions include nucleic acids which encode hRIP, hRIP kinase domains, and recombinant proteins made from these nucleic acids. The invention also provides methods for screening chemical libraries for lead compounds for a pharmacological agent useful in the diagnosis or treatment of disease associated hRIP activity or hRIP-dependent signal transduction. In one embodiment, the methods involve incubating a mixture of hRIP, a natural intracellular hRIP substrate or binding target and a candidate pharmacological agent and determining if the presence of the agent modulates the ability of hRIP to selectively phosphorylate the substrate or bind the binding target. Specific agents provide lead compounds for pharmacological agents capable of disrupting hRIP function.

## DETAILED DESCRIPTION OF THE INVENTION

A human RIP-encoding nucleic acid sequence is set out in SEQ ID NO: 1. A human RIP kinase domain-encoding nucleic acid sequence is set out in SEQ ID NO: 1, nucleotides 1-900. A human RIP amino acid sequence is set out in SEQ ID NO: 2; and a hRIP kinase domain sequence is set out in SEQ ID NO:2, residues 1-300.

Natural nucleic acids encoding hRIP are readily isolated from cDNA libraries with PCR primers and hybridization probes containing portions of the nucleic acid sequence of SEQ ID NO:1. For example, we used low stringency hybridization at 42°C (hybridization buffer: 20% formamide, 10 % Denhardt, 0.5% SDS, 5X SSPE; with membrane washes at room temperature with 5X SSPE/0.5% SDS) with a 120 base oligonucleotide probe (SEQ ID NO: 1, nucleotides 1728-1847) to isolate a native human RIP cDNA from a library prepared

from human umbilical vein endothelial cells. In addition, synthetic hRIP-encoding nucleic acids may be generated by automated synthesis.

The subject nucleic acids are recombinant, meaning they comprise a sequence joined to a nucleotide other than that to which sequence is naturally joined and isolated from a natural environment. The nucleic acids may be part of hRIP-expression vectors and may be incorporated into cells for expression and screening, transgenic animals for functional studies (e.g. the efficacy of candidate drugs for disease associated with expression of a hRIP), etc. These nucleic acids find a wide variety of applications including use as templates for transcription, hybridization probes, PCR primers, therapeutic nucleic acids, etc.; use in detecting the presence of hRIP genes and gene transcripts, in detecting or amplifying nucleic acids encoding additional hRIP homologs and structural analogs, and in gene therapy applications.

In a particular embodiment, the invention provides RIP-Thr<sup>514</sup> polypeptides, RIP-Thr<sup>514</sup> polypeptide-encoding nucleic acids/polynucleotides, and RIP-Thr<sup>514</sup> polypeptide-based methods (below), which RIP-Thr<sup>514</sup> polypeptides comprise at least 8, preferably at least 10, more preferably at least 12, more preferably at least 16, most preferably at least 24 consecutive amino acid residues of the amino acid sequence set forth as SEQ ID NO:2, which consecutive amino acid residues comprise the amino acid residue 514 (Thr) of SEQ ID NO:2. Exemplary RIP-Thr<sup>514</sup> polypeptides having RIP-Thr<sup>514</sup> binding specificity and immunologically distinguishable from RIP-Ser<sup>514</sup> are shown in Table I.

TABLE I. Exemplary RIP-Thr<sup>514</sup> polypeptides having RIP-Thr<sup>514</sup> binding specificity

$\alpha\Delta 1$ (SEQ ID NO:2, residues 509-518)	$\alpha\Delta 10$ (SEQ ID NO:2, residues 423-514)
$\alpha\Delta 2$ (SEQ ID NO:2, residues 514-521)	$\alpha\Delta 11$ (SEQ ID NO:2, residues 423-543)
$\alpha\Delta 3$ (SEQ ID NO:2, residues 506-514)	$\alpha\Delta 12$ (SEQ ID NO:2, residues 423-579)
$\alpha\Delta 4$ (SEQ ID NO:2, residues 504-524)	$\alpha\Delta 13$ (SEQ ID NO:2, residues 423-633)
$\alpha\Delta 5$ (SEQ ID NO:2, residues 498-514)	$\alpha\Delta 14$ (SEQ ID NO:2, residues 423-671)
$\alpha\Delta 6$ (SEQ ID NO:2, residues 514-534)	$\alpha\Delta 15$ (SEQ ID NO:2, residues 514-543)
$\alpha\Delta 7$ (SEQ ID NO:2, residues 513-520)	$\alpha\Delta 16$ (SEQ ID NO:2, residues 514-579)
$\alpha\Delta 8$ (SEQ ID NO:2, residues 508-515)	$\alpha\Delta 17$ (SEQ ID NO:2, residues 514-633)
$\alpha\Delta 9$ (SEQ ID NO:2, residues 512-522)	$\alpha\Delta 18$ (SEQ ID NO:2, residues 514-671)

In a particular embodiment, the invention provides RIP-ACA<sup>1540-1542</sup> polynucleotides, comprising at least 18, 24, 36, 48, 72, 148, 356 or 728 consecutive nucleotides of the nucleotide sequence set forth as SEQ ID NO:1, which consecutive polynucleotides comprise the polynucleotides 1540-1542 (ACA) of SEQ ID NO:1. Exemplary RIP-ACA<sup>1540-1542</sup> polynucleotides and allele specific oligonucleotide probes having RIP-ACA<sup>1540-1542</sup> binding specificity and distinguishable by hybridization assays from RIP-TCT<sup>1540-1542</sup> are shown in Table II.

TABLE II. Exemplary RIP-ACA<sup>1540-1542</sup> polynucleotides having RIP-ACA<sup>1540-1542</sup> binding specificity

$\alpha\Delta 1$  (SEQ ID NO:1, nucleotides 1540-1557)  
 $\alpha\Delta 2$  (SEQ ID NO:1, nucleotides 1540-1563)  
 $\alpha\Delta 3$  (SEQ ID NO:1, nucleotides 1540-1675)  
 $\alpha\Delta 4$  (SEQ ID NO:1, nucleotides 1540-1699)  
 $\alpha\Delta 5$  (SEQ ID NO:1, nucleotides 1525-1542)  
 $\alpha\Delta 6$  (SEQ ID NO:1, nucleotides 1519-1542)  
 $\alpha\Delta 7$  (SEQ ID NO:1, nucleotides 1507-1542)  
 $\alpha\Delta 8$  (SEQ ID NO:1, nucleotides 1483-1542)  
 $\alpha\Delta 9$  (SEQ ID NO:1, nucleotides 1537-1545)  
 $\alpha\Delta 10$  (SEQ ID NO:1, nucleotides 1534-1548)  
 $\alpha\Delta 11$  (SEQ ID NO:1, nucleotides 1528-1554)  
 $\alpha\Delta 12$  (SEQ ID NO:1, nucleotides 1516-1566)  
 $\alpha\Delta 13$  (SEQ ID NO:1, nucleotides 1504-1554)  
 $\alpha\Delta 14$  (SEQ ID NO:1, nucleotides 1492-1568)

The invention provides efficient methods of identifying pharmacological agents or lead compounds for agents active at the level of a hRIP modulatable cellular function, particularly hRIP mediated TNF receptor or Tumor necrosis factor receptor associated Factor -2 (TRAF2) or TRADD-induced signal transduction. For example, we have found that a binding complex comprising TNF R1, TRADD, and hRIP exists in TNF-stimulated cells. Generally, the screening methods involve assaying for compounds which interfere with a hRIP activity such as kinase activity or TRAF2 or TRADD binding. The methods are amenable to

automated, cost-effective high throughput screening of chemical libraries for lead compounds. Identified reagents find use in the pharmaceutical industries for animal and human trials; for example, the reagents may be derivatized and rescreened in in vitro and in vivo assays to optimize activity and minimize toxicity for pharmaceutical development. Target therapeutic indications are limited only in that the target cellular function be subject to modulation, usually inhibition, by disruption of the formation of a complex comprising hRIP and one or more natural hRIP intracellular binding targets including substrates or otherwise modulating hRIP kinase activity. Target indications may include infection, genetic disease, cell growth and regulatory or immunologic dysfunction, such as neoplasia, inflammation, hypersensitivity, etc.

A wide variety of assays for binding agents are provided including labeled in vitro kinase assays, protein-protein binding assays, immunoassays, cell based assays, etc. The hRIP compositions used in the methods are recombinantly produced from nucleic acids having the disclosed hRIP nucleotide sequences. The hRIP may be part of a fusion product with another peptide or polypeptide, e.g. a polypeptide that is capable of providing or enhancing protein-protein binding, stability under assay conditions (e.g. a tag for detection or anchoring), etc.

The assay mixtures comprise one or more natural intracellular hRIP binding targets including substrates, such as TRADD, TRAF2, or, in the case of an autophosphorylation assay, the hRIP itself can function as the binding target. In one embodiment, the mixture comprises a complex of hRIP, TRADD and TNFR1. A hRIP derived pseudosubstrate may be used or modified (e.g. A to S/T substitutions) to generate effective substrates for use in the subject kinase assays as can synthetic peptides or other protein substrates. Generally, hRIP-specificity of the binding agent is shown by kinase activity (i.e. the agent demonstrates activity of an hRIP substrate, agonist, antagonist, etc.) or binding equilibrium constants (usually at least about  $10^6 \text{ M}^{-1}$ , preferably at least about  $10^8 \text{ M}^{-1}$ , more preferably at least about  $10^9 \text{ M}^{-1}$ ).

A wide variety of cell-based and cell-free assays may be used to demonstrate hRIP-specific binding; preferred are rapid in vitro, cell-free assays such as mediating or inhibiting hRIP-protein (e.g. hRIP-TRADD) binding, phosphorylation assays, immunoassays, etc.

The assay mixture also comprises a candidate pharmacological agent. Candidate agents encompass numerous chemical classes, though typically they are organic compounds; preferably small organic compounds and are obtained from a wide variety of sources including libraries of synthetic or natural compounds. A variety of other reagents may also be included

in the mixture. These include reagents like salts, buffers, neutral proteins, e.g. albumin, detergents, etc. which may be used to facilitate optimal binding and/or reduce non-specific or background interactions, etc. Also, reagents that otherwise improve the efficiency of the assay, such as protease inhibitors, nuclease inhibitors, antimicrobial agents, etc. may be used.

5 In a preferred in vitro, binding assay, a mixture of at least the kinase domain of hRIP, one or more binding targets or substrates and the candidate agent is incubated under conditions whereby, but for the presence of the candidate pharmacological agent, the hRIP specifically binds the cellular binding target at a first binding affinity or phosphorylates the substrate at a first rate. After incubation, a second binding affinity or rate is detected.

10 Detection may be effected in any convenient way. For cell-free binding assays, one of the components usually comprises or is coupled to a label. The label may provide for direct detection as radioactivity, luminescence, optical or electron density, etc. or indirect detection such as an epitope tag, an enzyme, etc. A variety of methods may be used to detect the label depending on the nature of the label and other assay components. For example, the label may  
15 be detected bound to the solid substrate or a portion of the bound complex containing the label may be separated from the solid substrate, and thereafter the label detected.

The following examples are offered by way of illustration and not by way of limitation.

### EXAMPLES

20 1. Protocol for hRIP autophosphorylation assay.

A. Reagents:

- Neutralite Avidin: 20 µg/ml in PBS.

- hRIP:  $10^{-8}$  -  $10^{-5}$  M biotinylated hRIP kinase domain, residues 1-300 at 20 µg/ml in  
PBS.

25 - Blocking buffer: 5% BSA, 0.5% Tween 20 in PBS; 1 hour at room temperature.

- Assay Buffer: 100 mM KCl, 20 mM HEPES pH 7.6, 0.25 mM EDTA, 1% glycerol, 0.5% NP-40, 50 mM BME, 1 mg/ml BSA, cocktail of protease inhibitors.

- [ $^{32}$ P]γ-ATP 10x stock:  $2 \times 10^{-5}$  M cold ATP with 100 µCi [ $^{32}$ P]γ-ATP. Place in the  
4°C microfridge during screening.

30 - Protease inhibitor cocktail (1000X): 10 mg Trypsin Inhibitor (BMB # 109894), 10 mg Aprotinin (BMB # 236624), 25 mg Benzamidin (Sigma # B-6506), 25 mg Leupeptin

(BMB # 1017128), 10 mg APMSF (BMB # 917575), and 2mM NaVO<sub>3</sub> (Sigma # S-6508) in 10 ml PBS.

B. Preparation of assay plates:

- Coat with 120 µl of stock Neutralite avidin per well overnight at 4°C
- Wash 2 times with 200 µl PBS.
- Block with 150 µl of blocking buffer.
- Wash 2 times with 200 µl PBS.

C. Assay:

- Add 40 µl assay buffer/well.
- Add 40 µl biotinylated hRIP (0.1-10 pmoles/40 µl in assay buffer)
- Add 10 µl compound or extract.
- Add 10 µl [<sup>32</sup>P]γ-ATP 10x stock.
- Shake at 30°C for 15 minutes.
- Incubate additional 45 minutes at 30°C.
- Stop the reaction by washing 4 times with 200 µl PBS.
- Add 150 µl scintillation cocktail.
- Count in Topcount.

D. Controls for all assays (located on each plate):

- a. Non-specific binding (no RIP added)
- b. cold ATP to achieve 80% inhibition.

2. Protocol for hRIP - substrate phosphorylation assay.

A. Reagents:

- Neutralite Avidin: 20 µg/ml in PBS.
- hRIP: 10<sup>-8</sup> - 10<sup>-5</sup> M hRIP at 20 µg/ml in PBS.
- Blocking buffer: 5% BSA, 0.5% Tween 20 in PBS; 1 hour at room temperature.
- Assay Buffer: 100 mM KCl, 20 mM HEPES pH 7.6, 0.25 mM EDTA, 1% glycerol, 0.5% NP-40, 50 mM BME, 1 mg/ml BSA, cocktail of protease inhibitors.
- [<sup>32</sup>P]γ-ATP 10x stock: 2 x 10<sup>-5</sup> M cold ATP with 100 µCi [<sup>32</sup>P]γ-ATP. Place in the

4°C microfridge during screening.

- Substrate: 2 x 10<sup>-6</sup> M biotinylated synthetic peptide kinase substrate at 20 µg/ml in

PBS.

- Protease inhibitor cocktail (1000X): 10 mg Trypsin Inhibitor (BMB # 109894), 10 mg Aprotinin (BMB # 236624), 25 mg Benzamidine (Sigma # B-6506), 25 mg Leupeptin (BMB # 1017128), 10 mg APMSF (BMB # 917575), and 2mM NaVO<sub>3</sub> (Sigma # S-6508) in 10 ml PBS.

B. Preparation of assay plates:

- Coat with 120 µl of stock Neutralite avidin per well overnight at 4°C.
- Wash 2 times with 200 µl PBS.
- Block with 150 µl of blocking buffer.
- Wash 2 times with 200 µl PBS.

C. Assay:

- Add 40 µl assay buffer/well.
- Add 40 µl hRIP (0.1-10 pmoles/40 ul in assay buffer)
- Add 10 µl compound or extract.
- Shake at 30°C for 15 minutes.
- Add 10 µl [<sup>32</sup>P]γ-ATP 10x stock.
- Add 10 µl substrate.
- Shake at 30°C for 15 minutes.
- Incubate additional 45 minutes at 30°C.
- Stop the reaction by washing 4 times with 200 µl PBS.
- Add 150 µl scintillation cocktail.
- Count in Topcount.

D. Controls for all assays (located on each plate):

- a. Non-specific binding (no RIP added)
- b. cold ATP to achieve 80% inhibition.

3. Protocol for hRIP - TRADD binding assay.

A. Reagents:

- Anti-myc antibody: 20 µg/ml in PBS.
- Blocking buffer: 5% BSA, 0.5% Tween 20 in PBS; 1 hour at room temperature.
- Assay Buffer: 100 mM KCl, 20 mM HEPES pH 7.6, 0.25 mM EDTA, 1% glycerol,



0.5% NP-40, 50 mM  $\beta$ -mercaptoethanol, 1 mg/ml BSA, cocktail of protease inhibitors.

-  $^{33}\text{P}$  hRIP 10x stock:  $10^{-8}$  -  $10^{-6}$  M "cold" hRIP (full length) supplemented with 200,000-250,000 cpm of labeled hRIP (HMK-tagged) (Beckman counter). Place in the  $4^{\circ}\text{C}$  microfridge during screening

5        - Protease inhibitor cocktail (1000X): 10 mg Trypsin Inhibitor (BMB # 109894), 10 mg Aprotinin (BMB # 236624), 25 mg Benzamidine (Sigma # B-6506), 25 mg Leupeptin (BMB # 1017128), 10 mg APMSF (BMB # 917575), and 2mM  $\text{NaVO}_3$  (Sigma # S-6508) in 10 ml PBS.

- TRADD:  $10^{-8}$  -  $10^{-5}$  M myc epitope-tagged TRADD in PBS.

10      B.      Preparation of assay plates:

- Coat with 120  $\mu\text{l}$  of stock anti-myc antibody per well overnight at  $4^{\circ}\text{C}$ .
- Wash 2X with 200  $\mu\text{l}$  PBS.
- Block with 150  $\mu\text{l}$  of blocking buffer.
- Wash 2X with 200  $\mu\text{l}$  PBS.

15      C.      Assay:

- Add 40  $\mu\text{l}$  assay buffer/well.
- Add 10  $\mu\text{l}$  compound or extract.
- Add 10  $\mu\text{l}$   $^{33}\text{P}$ -RIP (20,000-25,000 cpm/0.1-10 pmoles/well =  $10^{-9}$  -  $10^{-7}$  M final

concentration).

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- Shake at  $25^{\circ}\text{C}$  for 15 minutes.
- Incubate additional 45 minutes at  $25^{\circ}\text{C}$ .
- Add 40  $\mu\text{l}$  epitope-tagged TRADD (0.1-10 pmoles/40  $\mu\text{l}$  in assay buffer)
- Incubate 1 hour at room temperature.
- Stop the reaction by washing 4 times with 200  $\mu\text{l}$  PBS.

25

- Add 150  $\mu\text{l}$  scintillation cocktail.
- Count in Topcount.

D.      Controls for all assays (located on each plate):

- a. Non-specific binding (no hRIP added)
- b. Soluble (non-tagged TRADD) to achieve 80% inhibition.

30

4.      Protocol for hRIP - TRAF2 binding assay.

A. Reagents:

- Anti-myc antibody: 20 µg/ml in PBS.
- Blocking buffer: 5% BSA, 0.5% Tween 20 in PBS; 1 hour at room temperature.
- Assay Buffer: 100 mM KCl, 20 mM HEPES pH 7.6, 0.25 mM EDTA, 1% glycerol,

0.5% NP-40, 50 mM β-mercaptoethanol, 1 mg/ml BSA, cocktail of protease inhibitors.

- <sup>33</sup>P hRIP 10x stock:  $10^{-8}$  -  $10^{-6}$  M "cold" hRIP kinase domain, residues 1-300, supplemented with 200,000-250,000 cpm of labeled hRIP kinase domain (HMK-tagged) (Beckman counter). Place in the 4°C microfridge during screening.

- Protease inhibitor cocktail (1000X): 10 mg Trypsin Inhibitor (BMB # 109894), 10 mg Aprotinin (BMB # 236624), 25 mg Benzamidine (Sigma # B-6506), 25 mg Leupeptin (BMB # 1017128), 10 mg APMSE (BMB # 917575), and 2mM NaVO<sub>3</sub> (Sigma # S-6508) in 10 ml PBS.

- TRAF2:  $10^{-8}$  -  $10^{-5}$  M myc epitope-tagged TRAF2 in PBS.

B. Preparation of assay plates:

- Coat with 120 µl of stock anti-myc antibody per well overnight at 4°C.
- Wash 2X with 200 µl PBS.
- Block with 150 µl of blocking buffer.
- Wash 2X with 200 µl PBS.

C. Assay:

- Add 40 µl assay buffer/well.
- Add 10 µl compound or extract.
- Add 10 µl <sup>33</sup>P-RIP kinase domain (20,000-25,000 cpm/0.1-10 pmoles/well =  $10^{-9}$  -  $10^{-7}$

M final concentration).

- Shake at 25°C for 15 minutes.
- Incubate additional 45 minutes at 25°C.
- Add 40 µl epitope-tagged TRAF2 (0.1-10 pmoles/40 ul in assay buffer)
- Incubate 1 hour at room temperature.
- Stop the reaction by washing 4 times with 200 µl PBS.
- Add 150 µl scintillation cocktail.
- Count in Topcount.

D. Controls for all assays (located on each plate):

- a. Non-specific binding (no hRIP kinase domain added)
- b. Soluble (non-tagged TRAF2) to achieve 80% inhibition.

5 All publications and patent applications cited in this specification are herein  
incorporated by reference as if each individual publication or patent application were  
specifically and individually indicated to be incorporated by reference. Although the foregoing  
invention has been described in some detail by way of illustration and example for purposes of  
clarity of understanding, it will be readily apparent to those of ordinary skill in the art in light  
of the teachings of this invention that certain changes and modifications may be made thereto  
10 without departing from the spirit or scope of the appended claims.